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Bordetella Extracytoplasmic Adenylate Cyclase: Structural and Functional Analogies with Bacillus anthracis Edema Factor Adenylate Cyclase (U)

Final Report

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Despite enzymatic similarities, the adenylate cyclase (AC) toxins from Bordetella pertussis and Bacillus anthracis appear to be very different structurally and functionally. The two do not compete in intoxication of target cells and clearly enter by different mechanisms, with EF + PA employing a receptor-mediated endocytosis pathway and B. pertussis AC toxin entering by unknown route. The two AC toxins exhibit some major differences in target cell specificities, with EF + PA being less efficacious in affecting immune effector cells such as monocytes. Although there appears to be a very limited immunologic cross-reactivity, the toxins do not seem to have significant structural homology. Examination of the two toxins in parallel has proven to be a useful approach, superior to the isolated study of either alone.

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FOREWORD

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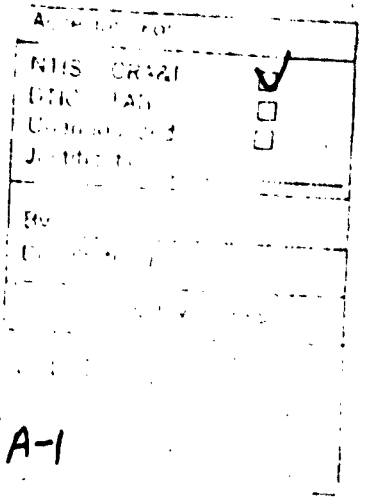

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I. Purification and characterization

Adenylate cyclase toxin, which possesses both enzymatic and toxin activities, is extracted from whole B. pertussis organisms because little or no toxin activity is released into the supernatant culture medium under the conditions of growth (1). Analogy with B. anthracis, however, does suggest that manipulation of the culture medium by addition of other materials (i.e. acetate buffer or serum) might promote the release of some enzyme/toxin during growth. Importantly, we have demonstrated that under apparently equivalent conditions, the ratio of cell-associated to soluble adenylate cyclase enzymatic activity for B. pertussis is almost the reciprocal of that for B. anthracis (Table I). Furthermore, addition of fetal calf serum markedly

Table I. Cell-associated and culture medium supernatant adenylate cyclase enzymatic activities of B. anthracis (Sterne strain) and B. pertussis (strain 114)

Organism	Medium	Adenylate Cyclase* (nmol cAMP/min/mg) Whole Cell	Supernatant
B. anthracis	BHI + FCS+	5.8	63.1
B. pertussis	BHI + FCS	20.5	0.8
B. pertussis	SS	924	300
B. pertussis	SS + FCS	1112	948

* In presence of 1 uM calmodulin
+ BHI - beef heart infusion; FCS - fetal calf serum, 10%; SS - synthetic Stainer-Scholte medium

potentiates enzymatic activity and increases the amount released into the medium by B. pertussis. Although adenylate cyclase toxin activities were not measured in these fractions, the data suggest that further modification of the system might facilitate toxin secretion by B. pertussis. It is of note also, that there is a significant fraction of residual cell-associated material in B. anthracis.

The extraction procedure for obtaining B. pertussis adenylate cyclase toxin from whole cells is as follows. Organisms are concentrated after 24 hours culture in Stainer-Scholte medium by centrifugation or filter concentration using Millipore Pellicon system. The resultant suspension of bacteria (120 ml from 7.2 liters of culture medium) is chilled to 4°C and urea is added with slow stirring to achieve a final concentration of 4M. The slurry is stirred overnight and the bacteria are then removed by centrifugation. There is less bacterial lysis and the extracted material has higher specific activity when the organisms are not frozen.

In the past, the urea extract was then dialyzed to remove the urea and loaded onto a hydrophobic chromatography resin, phenylsepharose. It is now clear that the enzyme/toxin will bind to the phenylsepharose in the presence of urea and that step is carried out by diluting the extract two-fold (to 2M urea) for loading. The column is then washed with 3M urea and eluted with 8M urea. Several observations indicate that the resultant material is in the form of a large aggregate or complex. First, when an aliquot is fractionated on a calibrated ACA-34 gel chromatography column, the enzyme/toxin activity all remains in the void volume (nominal molecular weight >350,000). This occurs whether or not the urea is removed by dialysis. This void volume fraction contains multiple (more than 20) bands on SDS-PAGE separation. Further purification is carried out by sucrose gradient centrifugation in an attempt to reduce toxin losses from adsorption to separation matrices. Urea is removed prior to sucrose gradient centrifugation by dialysis and concentration on collodion membranes (M_r=75K exclusion). Enzyme specific activity is often increased on this step without significant losses. The concentrate is fractionated on a 5-20% sucrose gradient at 40,000 rpm for 16-18 hrs. AC enzymatic and toxin activities are contained in a single peak which migrates with a calculated apparent size of M_r=68K (using MW markers of BSA, hemoglobin and catalase).

Attempts to fractionate the enzyme/toxin from the large complex by a variety of separation techniques including DEAE or hydroxyapatite chromatography, isoelectric focusing or sucrose gradient centrifugation have yielded inconsistent results with loss of activity and/or re-isolation of the unaltered aggregate (complex). With the hypothesis that the loss of activity might represent proteolytic degradation, protease inhibitors such as PMSF have been included in the purification buffers, but without objective effect.

Section II. Monoclonal antibodies

Without purified adenylate cyclase to use as antigen for preparation of monoclonal antibodies, a novel screening method had to be employed to detect positive clones. The pooled sucrose gradient peak (SG pool) has been used to prepare monoclonal antibodies (MAb) which have enabled identification and further purification of the AC toxin. Mice were immunized with SG pool and after the fusion (2), clones were screened initially in an ELISA against the same material. Antibodies from positive clones were evaluated for their ability to immunoprecipitate AC enyzmatic activity in the presence of protein A. Eleven clones, which were isolated and twice subcloned, continue to produce immunoprecipitating antibodies. Several of these antibodies (IgG_{2a} and IgG_1) have been prepared in mouse ascites, purified to the immunoglobulin fraction and coupled to activated CH-Sepharose 4B for use in immunoaffinity purification of the AC toxin. These antibodies uniformly identify a band of M_r=216K on Western blots of urea extract, phenyl-Sepharose pool, and sucrose gradient pool. In addition, there are several smaller molecular weight bands detected on Western blot suggestive of proteolytic cleavage products. When SG pool is used to further purify the AC toxin by immunoaffinity chromatography, the final produce has very high enzymatic and toxin activities. These monoclonal antibodies affect neither enzymatic nor toxin activity of the pertussis adenylate cyclase toxin. They have been evaluated for cross-reactivity with EF and PA (see below).

Section III. Comparison of adenylate cyclase toxins from B. anthracis and B. pertussis.

The majority of this work focused on the comparison of these toxins, with regard to their enzymatic activities, immunological and genetic cross-reactivities, their target tissue specificities and their effects on intoxicated cells. The relative specific activities are shown in Table II.

Table II. Adenylate cyclase activities of B. pertussis urea extract and B. anthracis purified edema factor (EF) and protective antigen (PA)

	Adenylate cyclase activity (umol cAMP/min/mg)	
	basal	+ CDR (1 uM)
B. pertussis urea extract	-	7.5
postphenylsepharose pool	0.003	39.3
sucrose gradient pool	0.081	42.4
affinity purified	0.180	125
B. anthracis		
edema factor	0.33	193
protective antigen	0.0001	0.05

Urea extract of B. pertussis not infrequently possesses specific activity of >10 umoles/min/mg, and this is either maintained or even reduced with initial purification (i.e. hydrophobic chromatography on phenylsepharose). These data suggest that degradation or loss of critical components may occur with this separation. Protection of the enzyme or reconstitution with other fractions has not, however, prevented or reversed this loss of activity. Purified edema factor (from Dr. Stephen H. Leppla, 3), on the other hand, has a specific activity of several hundred umoles/min/mg protein, with 500-1000-fold activation by calmodulin (CDR). In that a membrane-associated fraction of B. pertussis adenylate cyclase has been shown to be activated by phospholipid such as phosphatidylcholine (4), edema factor was evaluated for responsiveness to this activator. As shown in Table III, EF and its activation by calmodulin are unaffected by inclusion of uM PC in the reaction mix.

Table III. Effect of phosphatidylcholine, PA and anti-PA antibody on adenylate cyclase activity of EF

	Adenylate cyclase activity (umol cAMP/min/mg)			
	basal	+ CDR	+ PC	+ PC + CDR
Edema factor (EF)	0.11	124	0.24	130
EF + PA (undil)	0.13	55.2	N.D.	N.D.
+ PA (1:100)	0.21	132	4.8	99
EF + PA (undil) + anti-PA	0.25	71.8	N.D.	N.D.

Interestingly, while investigating the effect of PA on EF enzymatic activity, it was noted that basal (in the absence of calmodulin) activity of EF in the presence of PA is enhanced by PC.

In an attempt to assess whether EF interacts with PA in solution (prior to attachment to a target cell), the effect of PA on EF enzymatic activity was assessed. As shown also in Table III, PA in a three-fold excess over EF, but not at a 100-fold lower concentration, appeared to impair the enzymatic activity of EF. Anti-PA antibody was without effect under these conditions.

The monoclonal antibodies generated against sucrose gradient (SG) purified adenylate cyclase toxin were evaluated for immunologic cross-reactivity with EF and PA in an ELISA. The antibodies were in the form of hybridoma supernatants. In parallel, ascites-prepared monoclonals against EF or PA (provided by Dr. Stephen Leppla) were tested for reactivity with SG pool. Of the 21 anti-pertussis adenylate cyclase toxin antibodies, only one (9E9) reacted with PA and none with EF. Similarly, one anti-PA monoclonal (14C7) reacted with SG pool. The significance of this level of reactivity remains to be determined, but is intriguing in light of the recent demonstration of cross-reactivity of anti-B. pertussis adenylate cyclase antibody with human brain adenylate cyclase (5).

The original work of Leppla, demonstrating the adenylate cyclase toxin activity of EF and PA was conducted in Chinese hamster ovary (CHO cells) (3). One effect of EF and PA which was studied was the CHO cell elongation response to increased cAMP cells. In earlier studies of B. pertussis adenylate cyclase toxin, it had not been possible to elicit CHO cell elongation with urea extract containing adenylate cyclase, despite equivalent rises in cAMP levels to those achieved with EF and PA. The relationship between these responses was investigated and was very revealing. Figure 1 shows the CHO cell cAMP and elongation responses to increasing concentrations of EF and PA. While the dose-dependent cAMP accumulation is as expected, the

elongation response, reaching >30% of cells elongated, reaches a plateau between 0.003 and 0.03 ug/ml of EF and PA and declines

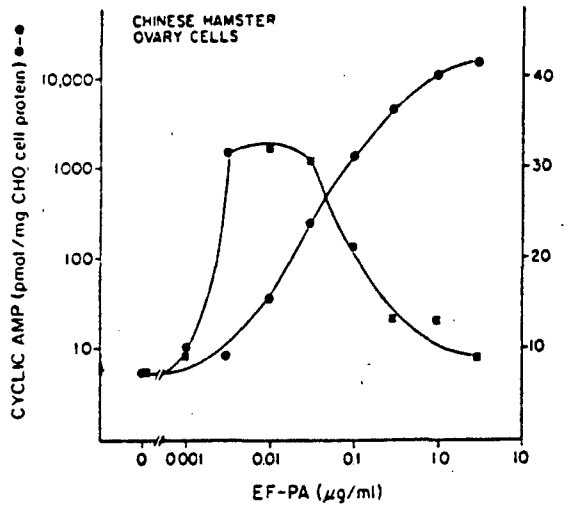

Figure 1. Cyclic AMP and morphologic responses of Chinese hamster ovary (CHO) cells to edema factor (EF) and protective antigen (PA). Cells were exposed to the indicated concentrations of toxins for 1 hour and cAMP extracted by 0.1 N HCl and assayed by RIA. Morphologic response was assessed in cells incubated with toxins for 18 hours.

thereafter. The B. pertussis extract had previously been tested only at maximal concentrations which, as with EF and PA, elicited cAMP of >10,000 pmol/mg protein. It is now apparent that at this level there is antagonism to the morphologic response, whether because of ATP depletion or cAMP-mediated toxicity. Addition of urea extract at lower concentrations now elicits elongation equivalent to that obtained with EF and PA (Figure 2). It is of note that only a very small increase in cAMP is required for the elongation to occur.

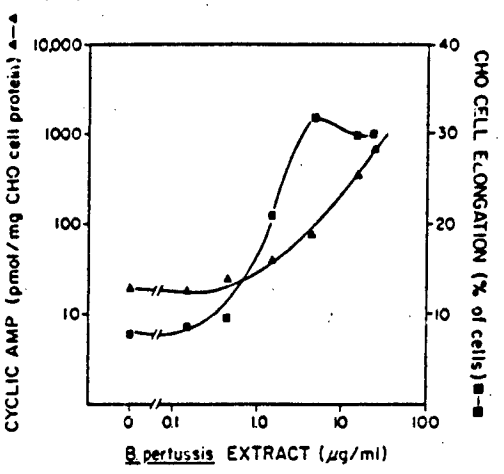

Figure 2. Cyclic AMP and morphologic responses of CHO cells to B. pertussis extract containing adenylate cyclase (AC) toxin. Cells were exposed to the indicated concentration of post phenyl sepharose pool for 1 hour, then cAMP extracted by 0.1 N HCl and assayed by RIA. Morphologic response was assessed in cells incubated 18 hours with extract.

S49 lymphoma cells have been useful in the study of B. pertussis adenylate cyclase toxin. The dose-dependent increase in cAMP levels is equivalent to that seen in CHO cells with EF and PA and there are mutants which possess defects in the endogenous adenylate cyclase of this cell. For these reasons, S49 cells were employed to compare the responses to the two adenylate cyclase toxins over a large concentration range. Unexpectedly, the efficacy of EF and PA in this cell is markedly reduced relative to their effects in CHO cells and to B. pertussis adenylate cyclase toxin effects in both cells (Table IV).

Table IV. Adenylate cyclase toxin activity of B. pertussis extract and B. anthracis EF + PA

Target cell	cAMP (pmol/mg prot) basal	B. pertussis extract	EF + PA
Chinese hamster ovary (CHO)	33 ± 4.8	14,360 ± 3570	11,500 ± 900
S49 lymphoma	6.2 ± 0.2	16,000 ± 1720	250 ± 60*
Human monocyte	20 ± 1.8	5,970 ± 288	733 ± 42+
Madin-Darby canine kidney (MDCK)			
Lines: AA7	10.9 ± 2	22.4 ± 5.9	21.9 ± 13+
BG11	8.6 ± 3.4	-----	9.5 ± 3.9
Transformed human lung fibroblast (VA-2)	4.1 ± 0.5	2,472 ± 656	661 ± 224*
Cervical carcinoma (HeLa)	3.1 ± 0.2	4.9 ± 0.6	4.3 ± 0.3*
Mouse B-cells			
Lines: DAUDI	15.0 ±	4,202 ± 96	-----
JY	5.6 ± 0.5	1,729 ± 476	198 ± 27.6*

Concentrations: Bp. extract 1:10 dilution; EF + PA, 3 ug/ml (*) or 10 ug/ml (+)

Not surprisingly, the potency of the purified EF and PA (ED_{50} ~ 1 ug/ml each) is greater than that of crude B. pertussis extract (ED_{50} ~ 100 ug/ml). The efficacy of the B. pertussis extract is, however, clearly greater by 100-fold. These observations suggest that there might be other differences in target cell sensitivities to those toxins. The comparative data collected to date are summarized in Table IV and depicted in Figure 3. Surprisingly, an equivalent response to the two toxins in CHO cells appears to be the exception rather than the rule (6). Importantly, one cell type (HeLa) has been identified which appears unaffected by either toxin (Table IV).

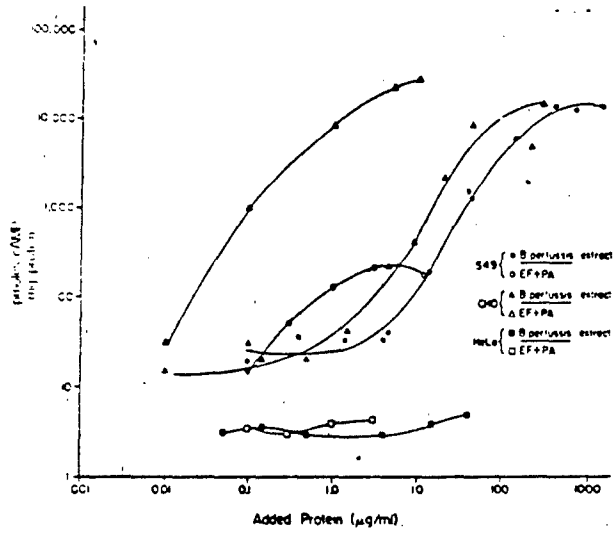

Figure 3. Effects of B. pertussis adenylate cyclase toxin and EF + PA on cAMP levels in different cell types. Toxins were added at the concentrations indicated with B. pertussis adenylate cyclase toxin in the form of urea extract. Cells were incubated at 37°C for 1 hour.

One major example that is perhaps of consequence pathophysiologically is the responsiveness of the human monocyte to these toxins. In Figure 4 is demonstrated the dose-dependent cAMP accumulation elicited by these toxins in human monocytes.

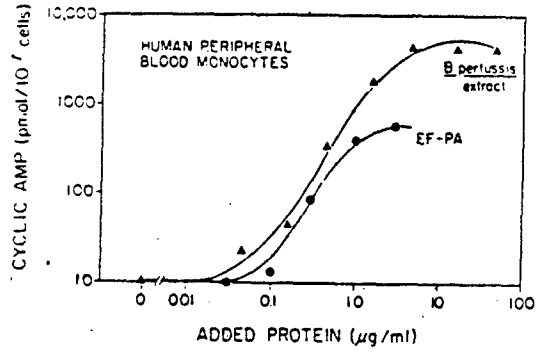

Figure 4. Dose dependence of cAMP accumulation in human monocytes elicited by EF and PA or B. pertussis AC toxin. Human peripheral blood monocytes were separated by sodium diatrizoate sedimentation and plastic adherence. The population was 65%-75% monocytes. Cells in suspension were incubated with EF and PA or B. pertussis extract at the indicated concentrations and the cAMP accumulation measured as above.

Interestingly, the two are almost equivalent in potency (ED_{50}s of 1-2 ug/ml), but B. pertussis AC toxin elicits 10-fold greater cAMP levels. The monocyte oxidative burst, as measured

by zymosan-induced chemiluminescence, is impaired by cAMP. The data in Figure 5 illustrate the inhibitory effect produced by cAMP from B. pertussis AC toxin, reaching total inhibition at an extract concentration of - 5 ug/ml with urea extract and 0.5 ug/ml with sucrose gradient pool.

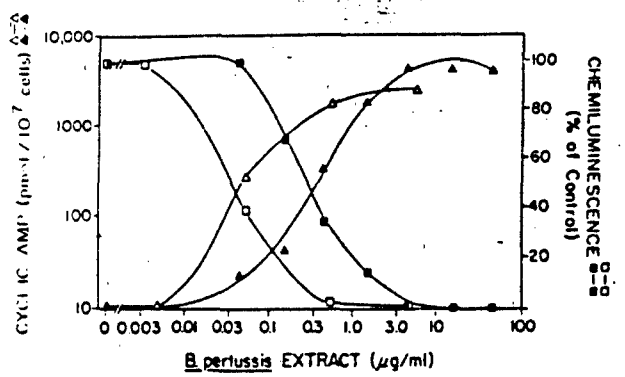

Figure 5. Effect of B. pertussis extract on monocyte cAMP levels and chemiluminescence repsonse. Human peripheral blood monocytes separated as described above were incubated with medium, urea extract of B. pertussis (closed symbols) or sucrose gradient pool (open symbols) for 1 hour. Cells were then either assayed for cAMP accumulation (triangles) or stimulated with opsonized zymosan (boxes). The chemiluminescence response to the zymosan stimulus was quantitated in a scintillation counter and results expressed as % of control (no B. pertussis extract).

As expected from the cAMP data, EF and PA are less efficacious in inhibiting the oxidative burst in these cells also (Figure 6).

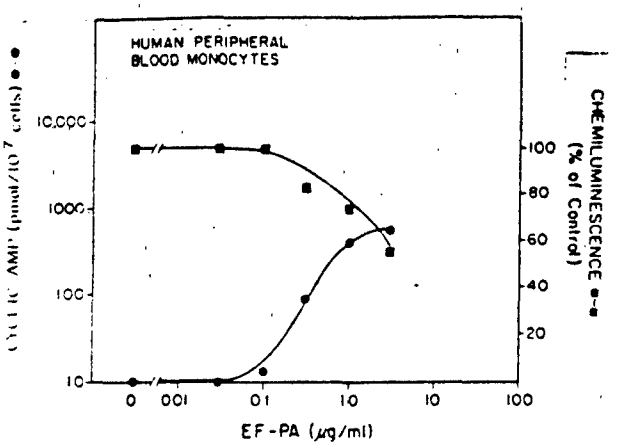

Figure 6. Effect of EF and PA on monocyte cAMP levels and chemiluminescence response. Conditions were as described for Figure 5 except that EF and PA, rather than B. pertussis extract, were added at indicated concentrations.

The differences in target cell specificities suggested that perhaps different receptors and/or entry mechanisms might be involved in intoxication by these different toxins. This has been addressed with the use of inhibitors of microfilament function and receptor-mediated endocytosis (7). Cytochalasin D, an inhibitor of microfilament function, abrogated the cAMP response to B. anthracis AC toxin (93%) but not the cAMP response elicited by B. pertussis AC toxin. B. anthracis-mediated intoxication of CHO cells was completely inhibited by ammonium chloride (30 mM) and chloroquine (0.1 mM), whereas the cAMP accumulation produced by B. pertussis AC toxin remained unchanged. The block of target cell intoxication by cytochalasin D could be bypassed when cells were first treated with anthrax AC toxin and then exposed to an acidic medium. These data indicate that despite enzymatic similarities, these two AC toxins intoxicate target cells by different mechanisms, with anthrax AC toxin entering by means of receptor-mediated endocytosis into acidic compartments and B. pertussis AC toxin using a separate, and as yet undefined, mechanism.

Polyclonal antisera against EF and against PA (provided by Dr. Stephen H. Leppla) have been evaluated for their ability to block toxin biological activity. When EF and PA are added at 0.01 ug/ml, anti-PA completely blocks CHO elongation at a dilution of 1:100 and partially blocks at 1:1000. The anti-EF, on the other hand, appears to be of lower titer, blocking the morphologic response only at 1:10. With a higher toxin concentration (0.1 ug/ml each), anti-PA totally blocks CHO cell cAMP accumulation at 1:500 and impairs it to 1:1000 while anti-EF doesn't even completely block at 1:50.

The kinetics of the EF and PA interaction has been examined in several experiments. Incubation of target CHO cells with PA for 30 minutes prior to addition of EF (1 ug/ml each), enhanced the response over addition of both together (Table V). When the prior incubation with PA was extended to 1.5

Table V. Effect of prior exposure to PA and removal of PA on the subsequent response of CHO cells to EF

First Addition (Time)	Wash	Second Addition (Time)	cAMP (at time 0) (pmol/mg/prot)
None	No	None	11.9
None	No	EF + PA* (-60 min)	17,700
PA (-90 min)	No	EF (-60 min)	25,654
PA (-90 min)	Yes	EF (-60 min)	14,416
None	No	EF + PA (-60 min)	17,686
PA (-150 min)	No	EF (-60 min)	17,369
PA (-150 min)	Yes	EF (-60 min)	10,154

* 1 ug/ml each

hours or longer this enhancement was lost, suggesting that prior attachment of PA might facilitate interaction with EF. Even removal of PA from the medium and washing the cells reduces the subsequent EF response by less than 50%. This concept is consistent with the hypothetical model put forth previously by Leppla (8), in which prior PA binding is required for EF to intoxicate the target cell. Whether EF binds to the attached PA remains unknown.

Section IV. Interaction of EF or PA with B. pertussis adenylate cyclase toxin

A major focus in this research has been the question of whether PA might be able to promote the entry of the Bordetella adenylate cyclase toxin as it does for EF. Since a preparation of B. pertussis adenylate cyclase with enzymatic activity but no toxin activity has not been available, this experiment has not been done. If these two toxins shared the same receptor for entry, however, one might expect that excess PA could impair the response to intact B. pertussis adenylate cyclase toxin. As shown in Table VI, however, that is clearly not the case. PA (5 ug/ml) does not affect the ability of B. pertussis adenylate cyclase toxin to increase cAMP levels in CHO cells. Furthermore, the cAMP response to the two toxins (B. pertussis adenylate cyclase toxin and EF plus PA) in CHO cells is additive (Figure 7).

Table VI. Effect of PA on cAMP accumulation in CHO cells elicited by B. pertussis extract (adenylate cyclase toxin)

	cAMP (pmol/mg prot)	
Addition	Control	+ PA (5 ug/ml)
None	3.3	N.D.
B. pertussis extract 1:10	1543	1569
1:100	42.3	50.6
1:1000	4.9	27

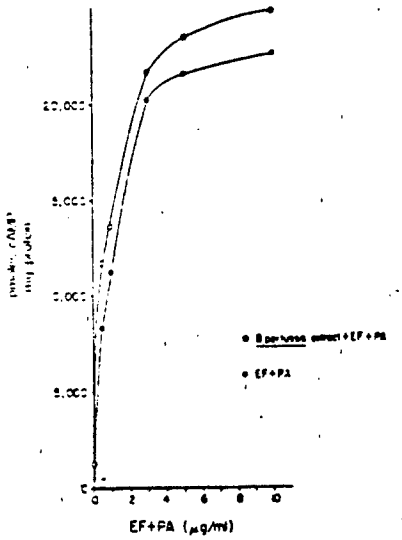

Figure 7. Effect of simultaneous addition of B. pertussis adenylate cyclase toxin in urea extract and EF + PA to CHO cells. Urea extract was added at a submaximal concentration (23 ug/ml) and EF + PA at the concentrations indicated. Incubation was for 1 hour at 37°C.

Conclusion

The accumulated data provide an initial picture of two toxins with apparently the same mechanism of action, namely introduction of a foreign adenylate cyclase enzyme into target cells to catalyze supraphysiologic cAMP accumulation. As with diphtheria toxin and pseudomonas exotoxin A, however, the bacteria of origin are quite different and despite the similar enzymatic properties of calmodulin activation and Ca^{++} inhibition the toxins appear to be quite different. Their target cell specificities and mechanisms of entry are different and the receptors appear to be different.

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